Cell, Vol. 98, 129–132, July 23, 1999, Copyright ©1999 by Cell Press

Evolving Ideas about Cyclins

Minireview

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The first molecular models of cell cycle regulation focused on how a single enzymatic oscillator, comprised of a B-type cyclin and a cyclin-dependent kinase (CDK) controlled mitotic entry and exit. However, these elegant and relatively simple models were soon recognized to be insufficient to account for the roles that CDKs play in multiple cell cycle events, including DNA replication, mitosis, centrosome/spindle pole body duplication, and cell morphogenetic changes.

Various explanations were then considered for how CDKs could control the orderly operation of the cell cycle, especially the regular alternation of S and M phases. An early debate was over whether the ability to induce temporally specific events was intrinsic to the CDK holoenzyme, or alternatively whether the changing state of the cell determined the particular cell cycle events that happened in response to CDK activation. Both factors probably contribute, but there is strong evidence that the CDK is critically important (Stern and Nurse, 1996 and references therein). Thus, premature CDK activation in G1 can cause a cell to skip S phase and proceed directly to mitosis. Other manipulations of CDK activity (for instance, turning the enzyme off and back on again) can cause a G2 cell to reenter S phase without an intervening mitosis.

The idea that biological specificity resides in the CDK holoenzyme itself presents a paradox. In yeast, a single CDK is responsible for initiating all cell cycle transitions (CDC28 in *S. cerevisiae* and CDC2 in *S. pombe*, referred to here generically as CDK1). What explains its specific effects at different times in the cell cycle? In higher eukaryotes the CDKs have evolved into small gene families whose individual members appear to be more important for one cell cycle phase or the other. CDK2, for instance, is associated primarily with activation of S phase whereas CDK1 primarily with mitosis. However, assays using defined peptides reveal only subtle differences in substrate preference between CDK1 and CDK2 (Holmes and Solomon, 1996), and there is no compelling evidence that CDK2 and CDK1 are intrinsically different with regard to their ability to initiate S and M phases. Indeed, when either of these genes are expressed in budding yeast they can rescue *cdc28* mutations, performing both its S and M phase functions (Elledge and Spottswood, 1991).

A different explanation for the specific actions of CDKs is that their regulatory subunits, the cyclins, are the major determinants of their biological specificity. All eukaryotes express multiple cyclins which assemble into complexes with CDKs in various combinatorial arrangements, controlling the patterns and level of CDK activity, and perhaps also playing roles in substrate selection (Figure 1A). Thus, each stage of the cell cycle can be characterized by its unique collection of cyclin–CDK complexes. It was not unreasonable to think that each different cyclin–CDK complex would execute distinct cell cycle events and that the sequential assembly and activation of these complexes could explain, at least in part, the order in which cell cycle events occur.

In fact, there is little doubt that different cyclins can have intrinsically different biological actions. On the one hand, there is a very clear distinction between the events that can be induced by the G1 versus S/G2 cyclins. In both budding and fission yeast, the S/G2 cyclins can initiate S phase and mitosis whereas the G1 cyclins cannot (Schwob and Nasmyth, 1993; Fisher and Nurse, 1996). Conversely, the G1 cyclins can perform functions that the B-type cyclins cannot, one example being the unique ability of the G1 cyclins in budding yeast to repress the mating pathway (Oehlen et al., 1998) and another the inability of the yeast B-type cyclins to activate the G1 transcriptional program (see Nasmyth, 1996 and references therein). Moreover, there are further functional divisions within these groups. The budding yeast G1 cyclins (the Cln proteins), which had been thought to be functionally redundant, turn out to be qualitatively

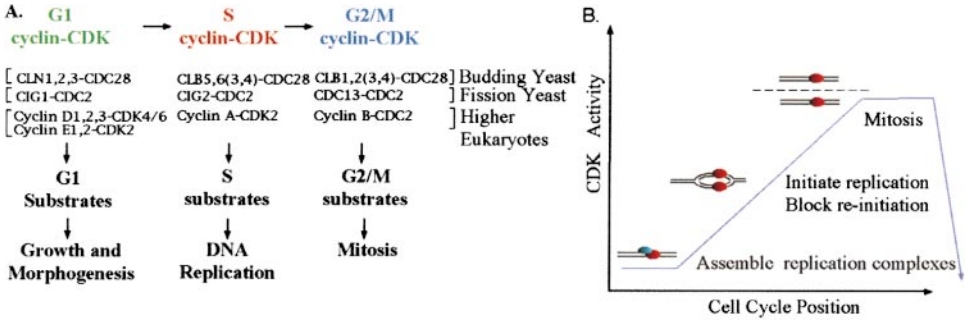

Figure 1. Qualitative and Quantitative Views of Cell Cycle Regulation

(A) The qualitative model. Special cyclins are needed for each phase of the cell cycle.

(B) The quantitative model. The figure depicts three CDK-modulated cell cycle events and represents how their execution may depend on the amount, not the type, of CDK activity. See text for details.

different in terms of the cell cycle events they stimulate (Levine et al., 1996). Although any one of the three CLN genes is sufficient for transit through G1, the particular pathways by which this happens depend upon whether it is Cln3p or Cln1/2p that is expressed. Cln3p seems to be an especially potent activator of the G1 transcriptional program, whereas Clns 1p and 2p can more directly activate G1 events such as bud emergence and cell morphogenesis.

The biological specificity of individual cyclins suggests that different cyclin-CDK complexes may have intrinsically distinct substrate preferences. Only a small number CDK substrates have been identified so its been difficult to establish the generality of this idea. But in the few cases where it has been tested, it seems that the ability of a CDK to phosphorylate a specific target protein depends on the particular cyclin to which it is bound. Some examples of this are that cyclin A–CDK1 and cyclin B–CDK1 phosphorylate histone H1 on different sites; that cyclin A–CDK2 but not cyclin B–CDK2 can phosphorylate the retinoblastoma-related protein p107; and that cyclin A–CDK2 but not cyclin E–CDK2 can phosphorylate the transcription factor DP1 (see Schulman et al., and references therein).

It is possible that cyclins affect CDK substrate choice by changing the structure of the CDK catalytic cleft, perhaps through allosteric interactions, thereby altering the affinity of the CDK for particular substrates. Indeed, the crystal structures of the CDK2 and cyclin A–CDK2 complexes show that binding of the cyclin to the CDK does alter the shape of the CDK both by reconfiguring the residues involved in ATP binding, and by repositioning the CDK T loop (the site of phosphorylation by the CDK-activating kinase) so that it does not completely obstruct the enzyme's catalytic cleft (Jeffrey et al., 1995). However, these changes facilitate phosphorylation of protein substrates in a general sense and do not seem to be involved in remodeling the catalytic cleft so that it would recognize one substrate better than another (Holmes and Solomon, 1996).

Another way that cyclins could contribute to substrate preference is by directly binding to substrates. This was first shown for the D-type cyclin–CDK complexes, whose efficient phosphorylation of the Rb protein is enhanced by an interaction between an LxCxE motif in the amino terminus of cyclin D and the Rb protein (Sherr, 1993). This motif is present in other proteins that bind directly and tightly to Rb, such as the E7 oncoprotein from papillomaviruses, and the E1A protein from adenoviruses. Cyclin E contains a similar motif that is also important for binding to, and phosphorylation of, Rb by cyclin E–CDK2.

The ability of cyclins to act as targeting subunits for the holoenzyme is not restricted to the special case of cyclin D binding to Rb. A second region involved in substrate docking is a conserved hydrophobic patch on the surface of the cyclin (Schulman et al., 1998). This surface groove was first seen in the cyclin A–CDK2–$p27^{Kip1}$ crystal structure as the site on the cyclin where the CDK inhibitor $p27^{Kip1}$ binds (Russo et al., 1996). The p27 residues that sit in this groove (the RxL motif) are conserved in other cyclin A–binding proteins, many or all of which are also CDK2 substrates including p107, p130, E2F, and even Rb. Moreover, mutations either in the hydrophobic patch or in the substrate RxL motif decrease both substrate binding and substrate phosphorylation. Indeed, the ability of cyclin A to induce entry into S phase is decreased by these same hydrophobic patch mutations, demonstrating a concordance between cyclin A biological specificity and substrate selectivity. The hydrophobic patch in cyclin A is also found in the D-, E-, and B-type cyclins. Therefore, this patch is unlikely on its own to be responsible for substrate selectivity, but may be a conserved substrate-docking domain whose selectivity is influenced by context and conformation.

A Quantitative Model for CDK Action

A very different perspective on cyclin specialization came from the work of Fisher and Nurse (1996), who reported that the cell cycle of the fission yeast *S. pombe* could run on a single B-type cyclin. A similar concept also emerged from Nasymth's lab based on experiments in budding yeast (Nasmyth, 1996), suggesting that complex models involving cyclin specialization might have missed an important underlying principle that could simplify our view of cell cycle regulation. There are three known fission yeast cyclins: Cig1, which plays a role in G1, Cig2, which is an S phase cyclin, and Cdc13, a B-type that is essential for mitosis. All three cyclins use CDK1 as their catalytic subunit. Deletion of Cig1 and Cig2 leaves Cdc13–CDK1 as the only known cyclin–CDK complex, and remarkably cells expressing just this one cyclin–CDK enzyme proliferate normally. These observations seemed to undermine the idea that qualitatively different cyclins are needed to control the regular alternation of S and M phases.

To account for these results a quantitative model for cell cycle regulation was proposed (Stern and Nurse, 1996) (Figure 1B). According to this model it is the amount of CDK activity that determines whether a cell will initiate S phase or mitosis, not the qualitative attributes of any particular cyclin–CDK complex. Moderate amounts of CDK activity would be sufficient to initiate S phase whereas higher amounts would be needed for mitosis. Alternation of S and M phases would be an automatic outcome of the steady rise and fall of a unitary CDK activity during each cell cycle.

A particularly appealing feature of this model is that it could neatly accommodate the additional regulatory requirements that mitosis not begin until S phase is finished, and that S phase be restricted to once per cell cycle. The coupling of mitosis to the completion of S phase could be accomplished by postulating that ongoing DNA replication inhibits the rise in CDK activity to mitotic levels. In this way mitosis would be held in check until S phase was completed. The second problem, that of preventing rereplication in a single cell cycle, could be dealt with by supposing that the initiation of DNA replication is a two-step process: first, loading of replication proteins at replication origins and second, triggering of those initiation complexes to begin DNA replication (see Nasmyth, 1996 and references therein). In order to make the model work it must then be the case that the first step is inhibited by CDKs and the second step activated by CDKs. Cells would then assemble initiation complexes at the conclusion of mitosis when the level of CDK activity is at a minimum, and then trigger those complexes to begin replication when CDK activity

increases to the appropriate level at the end of G1. The high amounts of CDK activity that persist for the remainder of the cell cycle would prevent the replication cycle from beginning again until CDK activity was restored to its basal state after the next mitosis.

Cyclin Specialization Revisited

Two new papers have reexamined the issue of cyclin specificity. Geng, Sicinski, and colleagues (Geng et al., 1999) compared the functions of the mammalian G1 cyclins D1 and E, and Cross and coworkers compared the budding yeast S/G2 cyclins CLB5 and CLB2 (Cross et al, 1999). The results from both groups strongly emphasize the idea that cyclins have specialized functions. The results from Cross et al. suggest that the quantitative view of cyclin function may not be widely applicable, and the results from Geng et al. sharpen our view of how cyclin D1 controls progression through G1.

The experiments of Geng et al. used knockout and knockin mice in which the *cyclin D1* locus was either deleted or replaced with cyclin E coding exons. Sicinski, together with Weinberg, had previously found that mice lacking the *cyclin D1* gene have specific deficits, including decreased proliferation of cells in the mammary gland and retina, and an undefined neurological abnormality (Sicinski et al., 1995). To explore the functional differences between cyclins D1 and E, they made homozygous mice in which the cyclin D1 genomic coding sequences were replaced with those encoding cyclin E. These mice make no cyclin D1, but instead transcribe a cyclin E message from the *cyclin D1* promoter (note that the normal *cyclin E* gene is still present in these mice, and its expression was not affected by the new *cyclin E→D1* gene). As a result, cyclin E protein is expressed with the correct developmental timing and tissue specificity of cyclin D1. This was found to rescue all of the known defects of the cyclin D1 knockout mouse. Also, no new phenotypes were associated with ectopic expression of cyclin E.

These results may be a surprise to those who have grown used to the idea that cyclin D1 and cyclin E have very different functions during G1. Indeed, at first glance, they may seem to support the idea that it is the timing of cyclin expression rather than their particular qualitative attributes that determines their biological functions. However, a key issue is whether cyclin E is performing the functions normally carried out by cyclin D1, or whether it is activating the cell cycle at a downstream step and bypassing the need for cyclin D1. Sicinski and colleagues suggest that the latter is more likely.

Cyclin D1 performs two known functions during G1. One is phosphorylation of Rb, which regulates the Rb/E2F transcriptional program. The other is sequestration of CDK inhibitors in the Kip/Cip family. Both of these have the effect of activating cyclin E, the first by inducing E2F-mediated *cyclin E* gene transcription, and the second by decreasing the inhibitory threshold imposed by the pool of free CDK inhibitors in the cell. Thus, there was reason to believe that activation of cyclin E may render the upstream roles of cyclin D1 unnecessary. In fact, transfection experiments in cultured cells had shown that ectopic expression of cyclin E would (at least in the short term) overcome cell cycle blocks imposed either by expression of a phosphorylation-deficient Rb protein (Leng et al., 1997; Lukas et al., 1997), or by overexpression of CDK inhibitors (Sheaff et al., 1997). When Rb phosphorylation and Kip/Cip sequestration are examined in the cyclin E→D1 knockin mice, it appeared that cyclin D1 functions were being bypassed by the ectopic cyclin E, not replaced; Rb phosphorylation did not proceed to completion in the knockin mice nor were the Kip/Cip proteins sequestered onto the ectopic cyclin E–CDK complexes. Therefore, the major conclusion of Geng et al.'s definitive work is that cyclin D1 and cyclin E act in sequence, and that cyclin E is the major downstream target of cyclin D1. What is happening to the rest of the E2F transcriptional program in these mice is not known, and it would be very surprising if this program were entirely dispensible when cyclin E is constitutively activated.

These results parallel the ones discussed above regarding the functions of the G1 cyclin genes in budding yeast. The D-type cyclins, like CLN3, are specialized for activation of the G1 transcriptional program whereas cyclin E, like CLN1/2 (or perhaps CLB5/6), is more involved in directly activating the downstream events of G1 progression (or entry into S phase). In yeast, as now seen in mammalian cells, ectopic expression of the downstream cyclin overcomes the need for the upstream one, although the actual pathways used to enter S phase are subtly different. Previous work had shown that expression of the *CLN2* gene from the *CLN3* promoter is sufficient for G1 progression (Levine et al., 1996), an observation that is quite analogous to the one reported by Sicinski.

One additional point deserves comment. Cyclin D1 had recently been proposed to have a CDK-independent function as a coactivator of the estrogen receptor (ER) (Zwijsen et al., 1997). It was suggested that this interaction might, in part, account for the hypoplasia of the mammary epithelium in the cyclin D1 knockout mice, and perhaps for hyperplasia of these cells in human tumors overexpressing cyclin D1. However, cyclin E does not interact with the ER. Therefore, rescue of mammary gland development in the E→D1 knockin mouse argues that cyclin D1's interaction with the ER is not important for understanding its actions in these cells.

Clear evidence for functional specialization among the B-type cyclins comes from the results of Cross et al. Budding yeast express six B-type cyclins among which CLB5 and -6 appear earliest in the cell cycle and are necessary for S phase, while CLB1 and -2 appear later and are required for mitosis. Cross replaced the chromosomal *CLB5* coding region with *CLB2*, thereby putting *CLB2* gene expression under control of the chromosomal *CLB5* promoter. This hybrid gene was functional, as it was able to rescue the lethality caused by deletion of CLB1 and -2. It was carefully shown that the timing of Clb protein expression and the amount of Clb-associated CDK activity closely corresponded to what happens in a normal yeast cell at the start of S phase. The only difference was that the actual cyclin–CDK complex being expressed was qualitatively different, containing Clb2p instead of Clb5p. Under these conditions it was found that Clb2p was extremely poor at promoting the start of S phase, demonstrating that Clb5p is somehow specialized for DNA replication. Prior work (Nasmyth, 1996) suggesting that CLB2 could initiate S phase relied on CLB2 overexpression and, therefore, could have

missed significant differences in efficiency between CLB2 and CLB5. The converse was also shown: that Clb5p cannot perform the mitotic functions associated with Clb2p. It was not determined whether the ability to prevent rereplication is a special property of Clb2p or Clb5p, but this is clearly an interesting question.

Further analysis showed that the specialized function of Clb5p required its surface hydrophobic patch, which is homologous to the one in cyclin A described above. Mutations in this patch had no effect on the ability of the Clb5p to bind to and activate CDK1, when this was assayed using histone H1 as a substrate, but substantially reduced its ability to promote S phase. Thus, it was not simply the amount of CDK activity but rather its particular quality that was important for S phase entry. This was also shown in another way. Mutations in Clb5p that interfered with CDK binding caused the expected decrease in protein kinase activity, when tested using the substrate histone H1. But these mutations did not decrease the potency of Clb5p for inducing DNA replication.

Retrospective

The qualitatively unique properties of individual cyclins are now supported by a wealth of genetic, biochemical, and molecular data. Nevertheless, the quantitative view of cyclin function has important implications when considering the evolution of the cell cycle. The observation that a single cyclin/CDK can orchestrate the entire *S. pombe* cell cycle led Nurse to suggest that this may represent an ancestral-like cell cycle. In primitive eukaryotes, with small genomes, chromosome replication and separation (S and M phases) may have been concurrent, and therefore could have been triggered by just one signal. Indeed, a single CDK target may have served the dual functions of acting as a site of replication initiation and as a locus for guiding chromosome separation. As genomes increased in size it would have become necessary to temporally separate replication from chromosome segregation, which could be accomplished by modifying the basic plan to allow S phase to be triggered first, by lower amounts of CDK activity, and M phase second, by higher amounts. Since the essential feature of this model is that the amount of CDK activity determines which cell cycle event occurs, one might suppose that the ancient function of the cyclin was to quantitatively control CDK activity. The multiple, qualitatively unique cyclins seen in modern organisms would be a fine-tuning mechanism, and not a fundamental attribute of the cell division process.

As an alternative, F. Cross has suggested that it may be useful to focus on the origin of cyclins as substrate-targeting subunits rather than as quantitative effectors of CDK activity. Perhaps ancient eukaryotes expressed proline-directed cyclin-independent enzymes that were the common progenitors of both cell cycle kinases (CDKs) and signal transduction kinases (MAPKs). These enzymes may have combined the functions of both pathways by responding to extracellular signals, like food, to simultaneously initiate cell growth and chromosome duplication. Cyclins may have first been used to facilitate substrate targeting by the ancestral "CDKs," functionally separating them from the signal transduction enzymes and ultimately from each other. The more familiar role of cyclins in modulating CDK activity might have come later, as CDKs evolved to become dependent on cyclins for their activity. This could have occurred as a fine-tuning mechanism designed to prevent indiscriminate protein phosphorylation by untargeted (free) CDKs.

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